Reproducibility of Methods Relating to Cyclophosphamide Metabolic Studies

The report by Boddy et al. (1) on the metabolism of cyclophosphamide once again highlights the complexity and difficulty involved in reproducing methods reported in the literature for investigation of this common therapeutic alkylating agent. Thus, Boddy et al. (1) found that not only did the reported procedure (2) require modification but also that one of the metabolites, nor-nitrogen mustard, could not be reliably measured. Here, we describe similar difficulties in repeating a procedure from the literature for the quantitation of phosphoramide mustard, an important and powerful intracellular alkylating metabolite of cyclophosphamide.

Juma et al. (3) reported that the specific and sensitive method they used to measure phosphoramide mustard in plasma was rapid and did not require expensive or sophisticated instrumentation. In a literature review, we found no studies in which this method had been applied subsequent to the original publication of the methods by Juma et al. When we attempted to repeat this assay, a number of limitations immediately became apparent, e.g., poor recoveries of phosphoramide mustard from plasma and inconsistent derivatization of the recommended internal standard. The attempted use of a structurally analogous internal standard, dechlorophosphoramide mustard, proved unsuccessful when it failed to form a derivative under the cited experimental conditions. Furthermore, some major changes in reagents (BCl3 or BF3) and the use of solvent homologues (methanol, ethanol, or propanol) had minimal effect on the reaction. These latter results implied a mode of reaction different from the simple transformations assumed from the published report of the study.

Juma et al. (3) proposed that the derivative of phosphoramide mustard they detected and measured was the O-methyl-N-trifluoroacetyl derivative. That derivative was not the product obtained when we replicated the methods of Juma et al. in subjecting synthesized O-methyl-phosphoramide mustard to trifluoroacetylation. More relevant, however, was the finding that cyclophosphamide and its metabolite 6-ketocyclophosphamide also form exactly the same derivative as phosphoramide mustard, while nor-nitrogen mustard and ifosfamide do not (Fig. 1). Compounds similar to phosphoramide mustard, such as O-methyl-phosphoramide mustard and phosphorotriamidate, also efficiently yield the same derivative. Thus, the reaction is not specific to phosphoramide mustard, and the potential exists for overestimation of phosphoramide or its metabolites have not been removed.

Our attempts to establish the identity of the derivative have been unsuccessful; therefore, it appears that the mechanism of formation is quite different from previously reported pathways for the transformation of cyclophosphamide and phosphoramide mustard (4-8). The fact that the reaction mechanism is unknown effectively precludes the choice of an appropriate internal standard. Therefore, within-sample correction for varying reaction efficiency or recovery is not feasible, which seriously limits the reliability and applicability of this method for quantitative studies.

Because of increased sensitivity in comparison with most published methods, however, this analytical method may have the potential to become a useful procedure for the quantitation of either cyclophosphamide or phosphoramide mustard if its mechanism can be delineated.

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References


Fig. 1. The reaction of cyclophosphamide, its metabolites, and ifosfamide using the procedure of Juma et al. (3). GC = gas chromatographic.
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Response

We are grateful for the opportunity to broaden the discussion regarding analytical methods for cyclophosphamide and its metabolites. The high-performance thin-layer chromatography–photography–densitometry assay originally described by Hadidi and Idle (1) can be successfully adapted to measure cyclophosphamide, dechloroethylcyclophosphamide, carboxyphosphamide, and 4-ketocyclophosphamide in plasma but does not reliably quantify phosphoramide mustard (2).

Several assay methods for phosphoramide mustard in plasma have been described (3-5). Some groups have tried to stabilize and isolate phosphoramide mustard as its methyl ester, using diazomethane. In our hands, this derivatization appears to be incomplete and therefore is hard to reproduce. We have tried the extraction technique described by Hardy et al. (6) but were unable to detect any phosphoramide mustard or any nonspecific alkylation activity in the extracts. A method involving a complex extraction for the analysis of isophosphoramide mustard, using phosphoramide mustard as an internal standard, has been described (7). This method appears to be sensitive and reproducible but is very laborious.

We have observed that, at room temperature in plasma, phosphoramide mustard degrades rapidly to non-nitrogen mustard, which in turn forms several minor breakdown products (Yule SM: unpublished results). This rapid deterioration makes sample preparation difficult. To overcome the difficulty of rapid deterioration, we have been rapidly freezing plasma samples and performing further manipulations at 5 °C. Plasma proteins are precipitated with cold acetonitrile, and the supernatant following centrifugation is then freeze dried at −40 °C. Thin-layer chromatography and detection are then performed as previously described (2). This method detects phosphoramide mustard in plasma at concentrations as low as 50 μM (Yule SM: unpublished results). However, this technique is not sufficiently sensitive to detect phosphoramide mustard in the plasma of patients receiving cyclophosphamide at doses below 60 mg/kg. This finding is in accordance with the results of Struck et al. (3) and Juma et al. (8) but conflicts with the findings of Moore et al. (9), who described phosphoramide mustard concentrations above 50 μM in patients receiving 40 mg/kg cyclophosphamide. Pharmacologic activity may be associated with phosphoramide mustard concentrations below this analytical threshold.

The relevance of plasma measurements of alkylating species to cytotoxic events within cells may be questioned. In vitro studies indicate that 4-hydroxycyclophosphamide is considerably more cytotoxic than phosphoramide mustard to tumor cells in culture (10, 11). This cytotoxicity is thought to be due to the high polarity of phosphoramide mustard, which is unable to penetrate cell membranes. Interestingly, an inverse relationship between plasma parent drug concentration and both toxicity and patient survival has been reported (12). Thus, while the determination of phosphoramide mustard concentrations in plasma may appear desirable, it may not be essential to determine the relationship between cyclophosphamide metabolism and clinical effect.

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References

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